Antimicrobial Activity of Stratum Corneum Lipids from Normal and Essential Fatty Acid-Deficient Mice

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Among the cutaneous effects of an essential fatty acid deficient (EFAD) diet are hyperdesquamation, increased transepidermal water loss (TEWL), and altered lipid profiles, characteristics also common to inflammatory dermatoses. Because fatty acids are antimicrobial, we examined the indigenous skin flora of normal and EFAD hairless mice, and compared the antimicrobial efficacy of lipids extracted from their stratum corneum. EFAD mice supported 100-fold more bacteria than normal mice, and were the only group from which *Staphylococcus aureus* were routinely isolated. Despite this greater carriage, in vitro experiments demonstrated that EFAD lipids are more lethal than normal lipids against *Streptococcus pyogenes, S. aureus, S. epidermidis, Micrococcus sp.,* and a coryneform. Skin fungi were equally susceptible to both extracts. After thin layer chromatography, the most active fractions were found to be glycosphingolipids and phospholipids. EFAD extracts had 35% more free fatty acids and 75% more glycosphingolipids; normal extracts had more triglycerides and phospholipids. *S. aureus* strain 502A survived equally well on EFAD as on normal mice. Normal lipids applied on EFAD mice had no additional effect, but EFAD lipids on normal mice brought about a 35% reduction of the inoculated bacteria. If the mice were pretreated with alcohol, carriage of strain 502A was reduced by 71%. If instead the mice were previously washed with acetone to increase TEWL, a 97% reduction of the staphylococcus occurred. The application of normal flora to such acetone-washed mice decreased the efficacy to 76%. EFAD and normal lipids on human subjects were equally ineffective in eliminating strain 502A. Results suggest that in vivo the normal flora regulates the killing of pathogenic microorganisms by potent EFAD skin lipids. *J Invest Dermatol 92:632–638, 1989*

Since the pioneering work of Burtenshaw [1], skin surface lipids have been regarded as effective antimicrobial factors against gram positive pathogenic bacteria that might attempt to colonize the stratum corneum and hair follicles [2,3]. These lipids have been thought to be the free fatty acids released from sebaceous triglycerides by lipases of the normal bacterial flora [4–6]. We recently re-examined the assumption that sebum-derived lipids are primarily responsible for this cutaneous antimicrobial activity. Our in vitro and in vivo studies involved extracts of human stratum corneum taken from abdominal sites in which sebaceous glands were relatively sparse. Results showed that endogenous, epidermis-derived free fatty acids, glycosphingolipids, and phospholipids also are effective against *Staphylococcus aureus* [7].

To assess further the role of endogenous stratum corneum lipids in antimicrobial defense, we compared the inhibitory activities of such lipids obtained from normal and essential fatty acid-deficient (EFAD) hairless mice. Essential fatty acid deficiency results in abnormal levels of transepidermal water loss, which is associated with a quantitative decrease in stratum corneum intercellular lipids [8]. The EFAD model resembles skin diseases such as psoriasis and atopic dermatitis by the presence of erythema, hyperdesquamation, and quantitatively and qualitatively altered stratum corneum lipid profiles [9–11]. Furthermore, in dietary essential fatty acid deficiency in humans and in acrodermatitis enteropathica, abnormal free fatty acids occur in both blood and skin, along with increased susceptibility to skin infections (especially candidiasis) [12,13].

Because inflammatory dermatoses in humans are often associated with infections and increased carriage of *S. aureus* [14,15], one would expect stratum corneum lipids of the EFAD mouse to be less effective against microorganisms than lipids extracted from its normal sibling. In the present study, we surveyed the indigenous flora of EFAD and normal mice, and found on EFAD mice, in agreement with this concept, both a higher population density of bacteria and the presence of *S. aureus*. However, subsequent in vitro and in vivo studies, which assayed extracted lipids, suggested the opposite conclusion: Stratum corneum lipids of EFAD mice, not normal mice, are significantly superior in antibacterial activity. We further noted that the benign normal flora of the mouse, also contrary to convention [16], assists the persistence of *S. aureus* applied to the skin.

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Abbreviations:

CFU: colony-forming units
EFAD: essential fatty acid-deficient
PBS: phosphate-buffered saline
TEWL: transepidermal water loss
TLC: thin layer chromatography
TSA: Tryptic soy agar
TSB: Tryptic soy broth

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MATERIALS AND METHODS

Animals and Experimental Diets Male hairless mice (Hr/Hr) (Jackson Laboratories, Bar Harbor, ME), aged 19–21 d and each weighing 6–12 g, were divided into two groups. The control group was fed ordinary Purina lab chow; the EFAD group received an isocaloric diet consisting of casein, sucrose, choline, a mixture of salts, fat-soluble vitamins A, D, and E, and water-soluble B vitamins to which 5% hydrogenated coconut oil and inositol (1 mg/g) were added. The composition of coconut oil was C9:0 5.4; C10:0 5.8; C12:0 48.3; C14:0 18.8; C16:0 9.8; C18:0 11.7, C18:1 0.2; C18:2 or C18:3 none detected (weight %). Clinical symptoms of essential fatty acid deficiency (fine scale, patchy erythema, wet and thickened skin, and small size relative to age-matched controls) became apparent in the EFAD group by 3–8 weeks. To monitor the onset and severity of the EFAD state, transepidermal water loss (TEWL) along the flank was measured weekly with a Meeco® electrolytic water analyzer, as described previously [17]. When the water loss exceeded 100 ppm/cm²/h (normal < 10 ppm), some of the EFAD and control mice were killed, and immediately whole skin samples were obtained for lipid extraction. Other animals from each group were reserved for the colonization experiments described below.

Survey of Mouse Normal Flora The population density and kinds of microorganisms inhabiting the skin of EFAD and normal mice were compared, using a modification of the Williamson and Kligman sampling technique [18]. Eight EFAD and eight normal mice were anesthetized with ether. A sterile copper collecting ring, 1.54 cm², pressed upon the abdomen of each mouse, and 1 ml of 0.075 M phosphate buffer with 0.1% Triton X-100 was added to the ring. The skin was then firmly rubbed for 1 min with a rounded metal spatula, and the buffer was removed with a sterile pipette. The process was repeated with a second milliliter of buffer. After pooling the fluid, 10^{-1} and 10^{-2} dilutions in half-strength buffer were prepared of each sample for inoculation of TSA, eosine methylene blue agar, and Sabouraud agar with chloramphenical and actidione. With the exception of Sabouraud agar plates, which were incubated at room temperature for 6 d, culture media were incubated at 37°C for 48 h.

Lipid Extraction Skin samples from four to eight mice were scraped free of all subcutaneous fat, and floated with dermis side downward on phosphate-buffered saline (PBS) containing 0.5% trypsin. After 2 h at 37°C, intact sheets of stratum corneum were slid off and floated again over fresh PBS-trypsin for 30 min to remove residual granular cells. The sheets were then subjected to vigorous vortex mixing in ice-cold PBS, and the turbid supernatant, containing individual stratum granulosum cells, was decanted. This procedure, when repeated several times until the supernatant becomes clear, provides stratum corneum sheets free of nucleated cells [19,20].

In a sequence found to remove over 99% of all stratum corneum lipids [20], the sheets were first lyophilized, weighed, and extracted for 18 h at 4°C in Bligh-Dyer solvents (chloroform:methanol:water, 2:4:1.6 vols) [20] at 1 ml of solvent/15 mg of dry tissue weight. The supernatant was set aside. Next, the remaining tissue was homogenized in Bligh-Dyer solvents in a glass tissue grinder, shaken in a modified Burrel wrist-action shaker for 10 min at 1,000 rpm, combined with the supernatant, centrifuged at 2,000 rpm for 10 min, and filtered through solvent-extracted filter paper (Whatman #43). To split the organic and aqueous phases, 2 ml of distilled water and 2 ml of chloroform were added to each 7.6 ml of lipid solution. The mixture was then shaken for 10 min and centrifuged for another 10 min. The upper (aqueous) phase was discarded and the lower (organic) phase was washed again with an equal volume of Bligh-Dyer upper phase aqueous solvents. The lower phase, containing all of the stratum corneum lipids, was dried under nitrogen gas in a 37°C water bath and stored in benzene or toluene at −16°C.

Thin Layer Chromatography Two-milligram samples of the total lipid extract from EFAD or control stratum corneum were applied to pre-cleaned 10 × 20 cm high-performance thin layer chromatography (TLC) plates (Merck HPTLC Silica Gel 60) [4-mg amounts on preparative 500-μm thick plates (Analtech Silica Gel G)], and fractionated sequentially in a neutral lipid solvent system (petroleum ether:diethyl ether:glacial acetic acid, 80:20:1 vols), a sphingolipid solvent system (chloroform:methanol:water, 90:10:1 vols or petroleum ether:diethyl ether:glacial acetic acid, 70:40:1 vols), and a polar lipid solvent system (chloroform:methanol:water:glacial acetic acid, 60:30:4.5:0.5 vols), as described previously [21]. Lipid standards (Sigma, St. Louis, MO), cofractionated, were TLC prepurified and maintained in stock solutions of 1 mg/ml in acetone at −16°C. After fractionation, the major lipids species were sprayed with 0.25% aqueous 8-anilino-1-naphthalene sulfonic acid and visualized under an ultraviolet A light source. Individual fractions were scraped off the plates, extracted with Bligh-Dyer solvents (see above), resuspended in benzene, and stored at −16°C. For phosphate analysis, full strength Phospray® was applied, and bands were visualized immediately at room temperature; for analysis of sugar moietites, orcine (200 mg of orcinol in 100 ml of sulfuric acid:water, 3:1 vols) was sprayed on, followed by 15 min incubation at 100°C.

Iatroscan Analysis For fractionation and quantification of small sample volumes, a microchromatographic technique was employed. Lipids were dried and resuspended in chloroform:methanol (2:1 vols) to a concentration of approximately 20 μg/ml. One microgram or less was spotted on each Chromarod, and the rod was developed in n-hexane:diethyl ether:formic acid (80:20:1 vols). The rods were then heated for 30 sec at 100°C to drive off remaining solvent and run through a flame ionization detector in the Iatroscan TH10-Mark III-TLC Analyzer (Ancal Corp., Los Osos, CA), using an atmospheric air flow of 2,000 ml/min, a high-purity hydrogen flow of 160 ml/min, and a scanning speed of 2.39 sec/cm. Detector response data were collected and integrated with a computing integrator model #SP4100. All of the Chromarods (Type SII) were pretested in appropriate solvent systems with known neutral lipid standards.

In Vitro Antimicrobial Assays Stock cultures of *Staphylococcus aureus* strain 502A, *S. epidermidis, Micrococcus sp., Streptococcus pyogenes,* an unclassified large-colony cutaneous coryneform (diphtheroid), and *Candida albicans* were maintained on Tryptic soy agar (TSA) (Difco) plates at 4°C. Spores of *Trichophyton mentagrophytes* were maintained in distilled water. Prior to testing, each microorganism was inoculated in Tryptic soy broth (TSB) (Difco) and incubated for 18 h at 37°C. The next day, 10^{-4} and 10^{-5} serial dilutions of each broth culture were prepared. Each series of assay tubes received 0.25 ml of peptone (Difco), 0.1 ml of acetone-dissolved lipid or, for control purposes, acetone only, and 0.1 ml of the microbial dilution. To disperse lipids and ensure that aggregation did not occur, tubes were repeatedly vortexed and observed. Tube-to-tube variation was always < 10%, and because acetone itself has a significant antimicrobial effect, triple acetone controls were used. Reactions were allowed to occur over 1 h; aliquots of the suspensions were then inoculated onto culture plates and examined 48 h later for colony quantification. We previously determined in similar assays that after lipid extraction and fractionation no free fatty acids were hydrolyzed from esterified lipids during these incubations [7].

In Vivo Antimicrobial Assays Groups of normal and EFAD mice, each comprising four to six animals, were used to examine the antimicrobial activity of unfractionated EFAD and normal stratum corneum lipids. Whatever the preliminary treatment, if any, between 220 and 250 μg of lipids in 20–30 μl of chloroform were delivered to a 3.5 cm² area of the right flank of the given mouse; an equivalent area of the left flank received a like volume of chloroform alone. Both flank sites were then inoculated with $5–8 \times 10^5$ colony-forming units (CFU) of *S. aureus* strain 502A in 0.01 ml of a 1:10 diluted 18-h culture. A sterile loop was used to distribute the inoculum on the skin. The incubation period of 5 h, besides being adequate for most host-lipid-microbial interactions, spanned the

time during which TEWL and epidermal lipid biosynthesis of acetone-washed mice (see below) most rapidly recovers to normal levels [22]. The surviving bacteria were harvested with a sterile calcium alginate swab moistened in 0.075 M phosphate buffer containing 0.1% Triton X-100. Although the cylinder-scrub technique [18] is somewhat more consistent and efficient than swabbing, it is more traumatic to the sensitive skin of mice. A further consideration in opting for swabs was that they could more easily cover the adjoining skin areas on the flank. This was important because the bacterium was likely to be spread beyond the initial site during the colonization period. The swab was then inserted in TSB containing 1.5% (w/v) sodium citrate, which dissolved the swab. One of the convenient attributes of *S. aureus* strain 502A is its tetracycline resistance; when samples were diluted in broth and cultured on TSA plates containing 5 μg/ml tetracycline, most of the normal flora of the mouse were inhibited, permitting easy quantification of the surviving inoculum.

Prior to application of the lipid, both flanks of some normal mice were washed with acetone-saturated, preextracted cotton swabs until TEWL exceeded 300 ppm/cm^2/h, as may occur in EFAD mice. Other groups of normal mice were treated with 70% alcohol wipes, ensuring that the alcohol was completely evaporated before inoculating the skin. Alcohol treatment did not alter TEWL. In one series of assays, after the skin was treated with acetone and lipid, both flank sites received 10^5 CFU of mixed indigenous flora 1 h before the application of strain 502A. The equal mixture of indigenous bacterial flora, previously obtained from these normal hairless mice, consisted of two representative staphylococci and three large-colony coryneforms.

Antimicrobial Assays of Mouse Lipids on Human Skin In vivo studies were also performed on five healthy human volunteers, who had not used germicidal skin products for at least 2 d and who had not received antibiotics for at least 2 weeks prior to testing. The flexor aspects of both forearms were rubbed with acetone-soaked cotton balls for 1 min; this procedure was repeated 4 times in order to remove most of the skin surface lipids. (Acetone washing does not disturb barrier function in humans.) Three 29-mm-diameter test sites then were delineated on each forearm with a sterile aluminum cylinder. Stratum corneum extracts from EFAD and control mice (0.1 ml at 3.5 or 0.35 mg/ml of acetone) were applied to the right and left forearms, respectively, inside a 1-cm diameter aluminum cylinder held firmly against the forearm. The lipid solution was spread evenly over the test sites and allowed to dry. A third test site, serving as vehicle control, received 0.1 ml of acetone alone. Immediately after the lipid applications had dried, each test site was inoculated with 10 μl of a diluted overnight culture of *S. aureus* strain 502A, containing about 5×10^3 CFU. The test sites were then covered with a small plastic weigh boat, and occluded with Tegaderm® (3M) transparent dressing. After 5 h, the cylinder scrub technique (see above), utilizing a sterile Teflon policeman and the 29-mm diameter ring, was used to harvest surviving bacteria. Again, the samples were plated on TSA containing 5 μg of tetracycline/ml to inhibit normal flora.

Analysis The in vitro microbiologic assays were performed at least in triplicate (a total of six series of dilution tubes). Antimicrobial activity was determined by comparing the colony counts of surviving microorganisms and of controls, and to standardize results, efficacy was expressed as percent reduction. In this simple system, each datum is normally distributed; probabilities of valid differences in activity between normal and EFAD lipids were calculated by the Student t test. In the in vivo antimicrobial assays, percent reductions were again based on arithmetic quantities, the colony counts from lipid-treated and control skin. However, to provide normality in distribution and thereby determine levels of statistical significance by the t test, a separate analysis was required in which the colony counts were transformed into logarithms.

RESULTS

Normal Flora of Mouse Skin In a coarse survey of normal flora residing on the skin of both EFAD and normal hairless mice, we found that the EFAD mouse carried 100-fold more bacteria than the normal mouse ($15.8 \pm 6.04 \times 10^5$ CFU/cm^2 vs $6.2 \pm 5.09 \times 10^3$/cm^2, $p < 0.01$). Furthermore, *S. aureus* was commonly found on EFAD mice (75% prevalence at $1.8 \pm 1.1 \times 10^3$ CFU/cm^2); it was not detected on normal mice. The gram positive flora consisted of the staphylococci, micrococci, and coryneforms common to mammalian skin. No gram negative bacteria or fungi were detected in either group of animals.

In Vitro Antimicrobial Assays Whole stratum corneum lipid extracts from EFAD and normal hairless mice were tested in vitro against a variety of normal and pathogenic cutaneous flora (Tables I and II). The differing antimicrobial activity of skin lipids did not directly reflect the results of the above survey. Although the normal lipid extract demonstrated comparatively low antibacterial activity, it was as effective as EFAD lipid against the fungi. EFAD lipids potently inhibited the pathogenic cocci *Staphylococcus aureus* and *Streptococcus pyogenes,* and to a slightly lesser degree, the normal

Table I. In Vitro Antibacterial Activity of Mouse Stratum Corneum Lipids

Lipid (mg/ml)	S. Aureus			S. Epidermidis			S. Pyogenes		
	EFAD	Normal	p	EFAD	Normal	p	EFAD	Normal	p
0.22	97 ± 4[a]	38 ± 15	<0.001	91 ± 6	19 ± 10	<0.001	85 ± 8	15 ± 4	<0.001
0.11	89 ± 12	26 ± 10	<0.001	69 ± 12	41 ± 14	<0.05	62 ± 12	16 ± 3	<0.001
0.056	92 ± 13	27 ± 16	<0.001	57 ± 12	23 ± 4	<0.001	43 ± 15	18 ± 6	<0.02
0.028	83 ± 17	20 ± 6	<0.001	34 ± 18	13 ± 3	<0.05	28 ± 10	6 ± 5	<0.01
0.014	46 ± 27	7 ± 4	<0.01	19 ± 8	15 ± 9	>0.10	16 ± 14	7 ± 4	>0.10
0.0069	26 ± 16	5 ± 5	<0.05	9 ± 9	5 ± 6	>0.10	4 ± 4	2 ± 1	>0.10
0.0034	6 ± 5	2 ± 2	>0.10	6 ± 2	5 ± 4	>0.10	—	—	—

Lipid (mg/ml)	Micrococcus			Coryneform		
	EFAD	Normal	p	EFAD	Normal	p
0.22	95 ± 4[a]	51 ± 16	<0.002	79 ± 17	32 ± 9	<0.005
0.11	92 ± 8	14 ± 10	<0.001	38 ± 10	25 ± 5	>0.05
0.056	66 ± 7	21 ± 10	<0.001	19 ± 6	17 ± 9	>0.10
0.028	42 ± 11	17 ± 9	<0.05	9 ± 2	9 ± 8	>0.10
0.014	29 ± 10	13 ± 9	>0.05	6 ± 5	8 ± 7	>0.10
0.0069	24 ± 14	7 ± 9	>0.10	—	—	
0.0034	12 ± 14	16 ± 11	>0.10	—	—	

[a] Percent reduction of colony-forming units ± SD; n = 6.

Table II. In Vitro Antimycotic Activity of Mouse Stratum Corneum Lipids

Lipid (mg/ml)	T. Mentagrophytes			C. Albicans		
	EFAD	Normal	p	EFAD	Normal	p
0.22	22 ± 4[a]	33 ± 10	>0.10	69 ± 19	72 ± 22	>0.10
0.11	9 ± 6	23 ± 6	<0.01	59 ± 23	55 ± 25	>0.10
0.056	24 ± 9	31 ± 9	>0.10	64 ± 17	44 ± 20	>0.10
0.028	12 ± 9	32 ± 4	<0.01	30 ± 26	35 ± 27	>0.10
0.014	8 ± 6	32 ± 19	<0.01	29 ± 8	33 ± 13	>0.10
0.0069	15 ± 8	11 ± 3	>0.10	7 ± 5	5 ± 6	>0.10
0.0034	15 ± 3	23 ± 6	>0.10	0 ± 0	2 ± 1	>0.10

[a] Percent reduction of colony-forming units ± SD; n = 6.

Table III. Anti-*S. aureus* Activity of Mouse Stratum Corneum Lipid Fractions

Lipid (mg/ml)	Neutral Lipids			Glycosphingolipids			Phospholipids		
	EFAD	Normal	p	EFAD	Normal	p	EFAD	Normal	p
0.22	98 ± 4[a]	74 ± 13	<0.002	100 ± 0	27 ± 11	<0.001	100 ± 1	13 ± 5	<0.001
0.11	93 ± 12	37 ± 6	<0.001	100 ± 0	16 ± 10	<0.001	96 ± 6	11 ± 6	<0.001
0.056	83 ± 37	25 ± 12	<0.01	100 ± 0	16 ± 14	<0.001	96 ± 6	4 ± 3	<0.001
0.028	80 ± 34	20 ± 8	<0.005	100 ± 0	5 ± 2	<0.001	94 ± 15	13 ± 2	<0.001
0.014	60 ± 34	13 ± 7	<0.01	100 ± 0	5 ± 4	<0.001	100 ± 1	11 ± 3	<0.001
0.0069	36 ± 26	25 ± 18	>0.10	100 ± 0	8 ± 8	<0.001	87 ± 26	15 ± 5	<0.001
0.0034	24 ± 24	14 ± 10	>0.10	98 ± 3	24 ± 14	<0.001	84 ± 30	18 ± 9	<0.005

[a] Percent reduction of colony-forming units ± SD; n = 6.

residents *Staphylococcus epidermidis* and *Micrococcus sp.* The coryneform was the least affected. While both lipid extracts showed moderate and uniform activity against *C. albicans,* the dermatophyte assay yielded inconsistent results.

S. aureus was then separately reacted with neutral lipid, glycosphingolipid, and phospholipid fractions obtained from both groups of mice. Of the normal lipids, only the combined neutral lipid fraction showed even moderate antibacterial activity (Table III). However, all of the fractions of EFAD stratum corneum lipids that were tested, particularly the glycosphingolipids and phospholipids, were highly effective, with 100% reductions found even in dilution tubes containing 3 μg of lipids/ml.

In Vivo Antimicrobial Assays in Mice In two independently conducted series of assays, we found no statistically significant difference between untreated EFAD and normal mice in carriage of the test strain of *S. aureus* (Table IV), despite indications from the above survey that EFAD mice are predisposed to colonization by *S. aureus.* Compared to untreated normal mice, a greater number of *S. aureus* survived on the acetone-washed animals. Because this effect could have been due to the removal of existing antimicrobial lipids, the perturbation of the skin barrier (high TEWL), the elimination of competitive normal flora, or a combination of the above, a variety of control experiments were performed. Alcohol-washed mice were also used, because alcohol did not affect TEWL and could not extract most of the skin lipids, yet both alcohol and acetone treatments reduced normal flora on normal hairless mice some 98% or 1.8 logs (alcohol, $p > 0.1$; acetone, $p = 0.01$). Data from the alcohol-washed mice were similar to those obtained from acetone-treated mice in demonstrating greater *S. aureus* carriage than untreated mice, but acetone treatment produced significantly higher levels. A more detailed analysis of alcohol treatment is given in Table V.

Next, we exchanged skin lipids between the two groups of mice (Table VI). Partial, variable effectiveness of EFAD lipids on normal mice was discernable, but results did not achieve statistical significance. However, EFAD lipids on alcohol-washed mice did significantly cause a reduction in test bacteria over the control site. The effect of EFAD lipids on acetone-washed normal mice was even more pronounced. Finally, the return of normal lipids to acetone-treated normal mice was ineffective. Again, as evidenced on the control sites, acetone washing allowed higher carriage of the staphylococcus. These results suggested that acetone washing or elevated TEWL by itself was not a critical factor in reducing carriage of test bacteria with EFAD lipids. We began to suspect that the elimination of resident flora was an important factor in the increased survival of *S. aureus.* Therefore, an additional experiment was performed to focus on the role of the normal flora. When acetone-washed mice were recolonized with normal flora prior to introducing EFAD lipid and *S. aureus,* net antistaphylococcal activity decreased.

In Vivo Antimicrobial Assays in Humans When EFAD or normal mouse stratum corneum lipids were applied to the forearms of volunteers, neither lipid extract demonstrated consistent activity against *S. aureus* (Table VII). This finding differed significantly from our earlier studies with human stratum corneum lipids performed under identical conditions [7].

Lipid Analysis The quantities and distributions of lipid species found in EFAD and normal mouse stratum corneum were compared. Analysis involved 12.6% dry weight samples of normal lipid and 10.2% dry weight of EFAD lipid. Although these quantities were not significantly different, we noted several important differences after fractionation (Table VIII). EFAD lipid contained 35% more free fatty acids and 75% more glycosphingolipids than fractions from normal mice, but one-half as much triglycerides. EFAD lipids also contained less than 1% phospholipids, compared with the

Table IV. Carriage of *S. Aureus* on Mice Skin without Lipid Pretreatment

Mouse	Arithmetic Mean[a]	Geometric Mean[a]	p[b]
Normal	$3.98 \pm 2.24 \times 10^3$	3.584 ± 0.129	—
EFAD	$4.42 \pm 2.24 \times 10^3$	3.601 ± 0.232	0.9
Acetone-washed normal	$7.95 \pm 3.56 \times 10^4$	4.838 ± 0.301	0.001
Alcohol-washed normal	$1.37 \pm 1.03 \times 10^4$	4.056 ± 0.326	0.43

[a] Data standardized as to an inoculum of 8×10^5 CFU/ml; n = 4 to 6.
[b] Compared with log counts from normal mice.

Table V. *S. Aureus* Carriage on Normal Mice After Alcohol Pretreatment

Site	Arithmetic Mean[a]	% Increase	Geometric Mean[a]	Log Increase
Treated	$6.87 \pm 5.16 \times 10^3$	49.5 ± 22.6	3.755 ± 0.326	0.335 ± 0.239
Control	$2.73 \pm 0.84 \times 10^3$	—	3.420 ± 0.150	—

[a] Colony-forming units ± SD; n = 4.

Table VI. In Vivo Anti-*S. Aureus* Activity After Lipid Pretreatment of Mice Skin

Lipid/Mouse/Treatment	Test Site[a]	Control Site[a]	% Reduction
Normal/EFAD	$3.13 \pm 3.51 \times 10^4$	$3.22 \pm 3.59 \times 10^4$	9.8 ± 17.3
EFAD/Normal	$1.49 \pm 1.39 \times 10^4$	$1.81 \pm 1.31 \times 10^4$	35.0 ± 35.8
Normal/Normal/Acetone	$7.81 \pm 2.60 \times 10^4$	$8.58 \pm 2.94 \times 10^4$	6.6 ± 17.6
EFAD/Normal/Acetone	$5.86 \pm 0.55 \times 10^3$	$2.22 \pm 1.40 \times 10^5$	96.8 ± 2.8
EFAD/Normal/Acetone + Flora	$4.90 \pm 4.91 \times 10^4$	$2.05 \pm 1.55 \times 10^5$	76.3 ± 9.3
EFAD/Normal/Alcohol	$7.24 \pm 6.50 \times 10^2$	$3.41 \pm 3.79 \times 10^3$	71.4 ± 12.9

Lipid/Mouse/Treatment	Test Site	Control Site	Log Reduction	p
Normal/EFAD	4.041 ± 0.872	4.092 ± 0.825	0.052 ± 0.098	$p > 0.9$
EFAD/Normal	3.857 ± 0.682	4.131 ± 0.382	0.270 ± 0.313	$p = 0.4$
Normal/Normal/Acetone	4.873 ± 0.147	4.909 ± 0.158	0.010 ± 0.056	$p > 0.7$
EFAD/Normal/Acetone	3.538 ± 0.562	5.257 ± 0.350	1.719 ± 0.594	$p = 0.002$
EFAD/Normal/Acetone + Flora	4.527 ± 0.415	5.186 ± 0.407	0.659 ± 0.187	$p = 0.06$
EFAD/Normal/Alcohol	2.728 ± 0.354	3.343 ± 0.426	0.615 ± 0.319	$p = 0.01$

[a] Colony-forming units ± SD; n = 4 to 6.

2% present in normal stratum corneum. Two other such analyses provided similar data (not shown).

Analysis of TLC plates demonstrated further differences between the two lipid extracts. Whereas the number of bands and staining patterns of normal lipids run in the glycosphingolipid solvent system resembled those found in previous studies [19], orcine staining of EFAD lipids revealed additional sugar-containing species. Thus, the increased free fatty acid content and particularly the composition of polar lipids of EFAD stratum corneum make this animal model particularly distinctive.

DISCUSSION

This study revealed clear microbiologic differences between EFAD and normal hairless mice in the quantity and types of normal flora and in the antibacterial activity of extracts and fractions of stratum corneum lipid both in vitro and in vivo. However, numerous questions and apparent paradoxes arise from the data.

The superior potency of EFAD lipids was related not only to the free fatty acids, as expected [1,4,5], but also and especially to the glycosphingolipid and phospholipid fractions. The last two lipid species were previously found to be antistaphylococcal, but to a lesser degree than free fatty acids, in extracts of normal human stratum corneum taken from fresh cadavers [7]. The proportions of fatty acids and glycosphingolipids in these human extracts are similar to those found in EFAD mice. Human stratum corneum, however, contains about twice as much phospholipid (4% vs 1%–2% weight) and a far smaller proportion of sterol esters (1% vs 18%–19% weight) than either group of mice. The smaller percentage of fatty acids and glycosphingolipids in the stratum corneum of normal mice is not the entire explanation for its relatively weak antimicrobial activity, because lower potency was also evident when we tested equal concentrations of the respective fractionated species. Gas-liquid chromatography or a similar tool may help resolve the intra-species differences between the normal and EFAD lipid fractions in mice. Recently, such analyses have been performed on lipids from EFAD rats and pigs, which in rats revealed increases in the relative amounts of glucosylceramides, acylglucosylceramides, and acylceramides with a decrease in one ceramide fraction, and in both animals the replacement of linoleates by oleates [23,24].

Why EFAD mice support greater populations of skin flora, particularly *S. aureus,* than normal mice when their lipids are so much more lethal in vitro is the foremost ecologic question. Even in the artificial situation, inocula of *S. aureus* survived equally as well on EFAD mice as on normal animals. While the superior activity of EFAD lipids would seem to be only an in vitro phenomenon, two early pilot studies using *S. aureus* or *S. pyogenes* as inocula suggested that the EFAD mouse was indeed more antibacterial (unpublished data). In our present in vivo studies, concentrations of tested lipid were of physiologically relevant amounts [7], some 10 to 100 times more than calculated to exist in intercellular domains of the stratum corneum. Furthermore, the assay incubation periods should have been adequate for the inoculated bacteria to adjust to their new environment and interact with the lipids. Any possible differences in the form and availability of these lipids in vivo (experimentally

Table VII. In Vivo Anti-*S. Aureus* Activity of Mouse Stratum Corneum Lipids on Human Skin

	EFAD Lipids[a]			Normal Lipids		
Subject	3.5 mg/ml	0.35 mg/ml	Vehicle Control	3.5 mg/ml	0.35 mg/ml	Vehicle Control
1	1.42[b]	2.48	2.29	3.28	3.00	3.18
2	0.41	0.28	0.30	0.17	0.09	0.09
3	2.93	1.67	1.40	2.02	1.76	1.60
4	1.72	2.10	2.28	2.54	1.90	2.18
5	1.08	0.76	1.24	1.63	1.46	1.80
Mean	1.51	1.46	1.50	1.93	1.63	1.77

[a] 0.1 ml of lipid in acetone applied to 0.79 cm^2.
[b] 10^3 CFU/cm^2.

Table VIII. Fractionation of Hairless Mouse Stratum Corneum Lipids

Fraction	Lipid Weight %	
	EFAD	Normal
Neutral lipids		
Hydrocarbons	2	2
Sterol esters	19	18
Triglycerides	6	13
Free fatty acids	27	20
Free sterols	20	17
Unknown	4	7
Polar lipids		
Ceramides	17	18
Glycosphingolipids	7	4
Phospholipids	1	2
Total recoveries	103	101

applied) versus in vitro or in situ, i.e., free vs. organized in membranes, should not have changed the observed imparities in antibacterial activity. The solution to the inconsistency appears to lie in the preparatory treatment of the normal mice.

The intent of subjecting normal mice to acetone washes was to reproduce both the TEWL and enhanced epidermal lipogenesis of EFAD mice [22]. Acetone also would remove most surface lipids, providing a clean slate for the application of test lipid extracts. Under these conditions, we found EFAD lipids to be significantly efficacious, echoing the in vitro results. Subsequent variations of experimental design, which in some cases involved treatment with the more gentle alcohol washes, determined that neither increased TEWL nor the removal of existing lipids by itself was the sole cause of the phenomenon. Acetone treatment alone even seemed to support carriage during the test period. The more important factor, next to EFAD lipids, was apparently a side-effect of acetone: the elimination of normal skin flora. Maximal antistaphylococcal activity was present when both endogenous surface lipids and indigenous flora were removed prior to application of EFAD lipid. Thus, the antimicrobial activity of the skin can be regarded as a complex interactive, synergistic action of several components, not of a single agency. Furthermore, the peculiar in vivo phenomenon, indeed both the carriage of pathogens and the maintenance of normal flora, requires a dynamic equilibrium of growth promoting and inhibiting processes.

We were keenly reminded of the importance of in vivo ecologic studies when the contribution of normal flora was considered in the assay system. EFAD lipids in our in vitro assays showed the least activity against the *Micrococcus* isolate and the coryneform. While less resistant than these common skin commensals, *S. epidermidis*, nevertheless, survived better than the sometime pathogenic resident *S. aureus*. What molecular mechanisms enable normal flora to resist the lethal effects of these fatty acids and polar lipids, and how these lipids kill transients and pathogens are poorly understood. Various strategies may be involved, depending on the particular bacterium. Some coryneforms are actually lipophilic and secrete lipases and esterases [25, 26]. Such enzyme production often occurs in the Micrococcaceae, including *S. aureus* [27,28]. However, we did not detect lipid hydrolysis and release of fatty acids within the 1-h incubation of our in vitro testing. Microbial interactions affecting extracellular lipid digestion also have been observed: certain *Micrococci* produce a factor that inhibits coryneform esterase activity [29]. Microbial lipases may also be inhibited by fatty acids [30]. Because resistance of normal flora to skin lipids is not absolute, low membrane ligand avidities may be involved. Regardless of the various molecular devices involved in microbial resistance, skin lipids are an important selective influence in the composition of normal flora.

Another colonization factor that was probably affected by the test procedures is selective microbial adherence [31]. Vigorous acetone washing could have removed fully keratinized epithelial cells, which carry greater densities of binding sites for a variety of microorganisms [31]. Consequently, the loss of these receptors would have reduced carriage of the inoculated staphylococcus. Alternatively, the removal of competitive normal flora from such sites would have provided a greater opportunity for *S. aureus* to attach to the kertinocytes [32]. Because acetone treatment enhanced survival of the inoculated bacteria compared to untreated mice, the second possibility appears more likely.

How then does *S. aureus* colonize EFAD mice? How, in the key experimental variation, does *S. aureus* endure longer on acetone-washed normal mice in the presence of EFAD lipid and normal flora than on mice without normal flora? No simple, precise answers are available to these ecologic questions. In the first instance, normal flora, which are present in large populations, may utilize, degrade, or otherwise neutralize the lethal lipid species. The high humidity and seepage of electrolytes [8] and other low molecular weight substances due to the perturbed barrier supports bacterial growth, and the mild inflammation of the skin, which probably provides additional nutrients, more binding sites, and other supportive conditions, predisposes to *S. aureus* colonization. In the experimental situation, the temporarily perturbed barrier provides an environment also supportive of bacterial colonization. The reintroduced normal flora again protects the staphylococcus from the effects of lipids, although these residents occupy many of the attachment sites on keratinocytes. The data from other experimental conditions may be explained by similar combinations of these dynamic factors.

The inability of EFAD lipids to cause a greater reduction of the *S. aureus* inoculum on the skin of human volunteers than normal mouse lipids or even the vehicle is not so easily understood. Human skin is far more refractory than mice to acetone-induced changes in barrier function. Perhaps the volunteers lacked some necessary cofactor that could have percolated through the stratum corneum in mice. Some inhibitor of EFAD lipids unique to human skin is also possible, but it would not be proper to make much of the single trial and small number of subjects. What is clear, however, is that EFAD mice and their lipids should be the subject of further microbiologic investigations.

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